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Chapter 6

PREERYTHROCYTIC MALARIA VACCINE DEVELOPMENT*

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I. FOUNDATION FOR PREERYTHROCYTIC MALARIA VACCINE DEVELOPMENT: THE IRRADIATED SPOROZOITE MODEL

In the 1940s, Mulligan and colleagues[1] demonstrated that immunization of chickens with radiation-attenuated *Plasmodium gallinaceum* sporozoites induced protective immunity. In the late 1960s, Nussenzweig and colleagues[2] demonstrated that immunization of A/J mice with radiation-attenuated *P. berghei* sporozoites protected mice against challenge with live sporozoites. This immunity was stage specific; mice challenged with infected erythrocytes were not protected. In the early 1970s Clyde and colleagues[3-5] and Rieckmann and colleagues[6,7] demonstrated that immunization of humans by the bite of irradiated *Anopheles* sp. mosquitoes carrying *P. falciparum* and in one case *P. vivax* sporozoites in their salivary glands protected these volunteers against challenge with live sporozoites. As the immunity in mice, this immunity was stage specific, and it was also species specific; immunization with *P. falciparum* did not protect against *P. vivax*. However, it was not strain specific; immunization with *P. falciparum* sporozoites from Burma protected against challenge with sporozoites from Malaya, Panama, and the Philippines,[3] and immunization with sporozoites from Ethiopia protected against challenge with a strain from Vietnam.[7] These human studies have been repeated recently,[8,9] reconfirming that there already is an effective malaria vaccine and demonstrating this protective immunity lasts for at least 9 months.[9a] Unfortunately, sporozoites have to be delivered alive; and since mature, infective sporozoites have never been produced *in vitro* and it is impractical to immunize large numbers of individuals by the bite of thousands of sporozoite-infected mosquitoes, the targets and mechanisms of this protective immune response had to be identified so as to construct a synthetic or recombinant vaccine.

Radiation-attenuated sporozoites develop only to late trophozoites in the liver; and this observation and the finding that irradiated sporozoite-induced immunity does not protect against challenge with infected erythrocytes, indicate that the immunity is directed against the sporozoite as it rapidly makes its way from inoculation by the mosquito to the hepatocytes, or against the infected hepatocyte. Since the sporozoite is primarily extracellular during the 60 min or less that it takes to invade hepatocytes,[10] antibodies may prevent sporozoites from effectively in-

vading hepatocytes; and either antibodies or T cells could recognize parasite antigens expressed in infected hepatocytes and destroy these cells. Initial efforts to develop preerythrocytic malaria vaccines focused on producing protective antibodies. Currently there is increasing recognition of the requirement to attack the infected hepatocyte, primarily through T cell-mediated mechanisms.

II. PREVENTING SPOROZOITES FROM EFFECTIVELY INVADING HEPATOCYTES

A. RATIONALE

Sera from mice and humans immunized with irradiated sporozoites precipitate the surface coat of live sporozoites; this is known as the circumsporozoite (CS) precipitation reaction.[3,5,11] It seemed logical therefore that antibodies in these sera mediated this CS precipitation reaction rendering sporozoites noninfectious, and thereby preventing malaria. In 1980 Potocnjak[12] reported that passive transfer of Fab1 that recognized the 44-kDa *P. berghei* CS protein mediated the CS precipitation reaction, and conferred protection against sporozoite-induced *P. berghei* infections. Passive transfer of monoclonal antibodies against the *P. yoelii*[13,14] and *P. vivax*[15] CS proteins has also been shown to protect against sporozoite-induced malaria. Since these antibodies have no effect when passively transferred 5 min after sporozoite inoculation,[15a] it is thought that they recognize sporozoites in the circulation and prevent them from effectively invading hepatocytes. The mechanism of this action is unknown; however, since it can be mediated by Fab1[12] and is active in mice depleted of complement by prior injection with cobra venom and in Balb/c nu/nu mice who do not have T lymphocytes,[15b] it apparently requires only the interaction of sporozoite with antibody and is independent of other immune effector mechanisms.

The genes encoding numerous *Plasmodium* sp. CS proteins have been cloned and sequenced. All CS proteins characterized thus far have a central region of tandemly repeated amino acids. In *P. falciparum* NANP is repeated 35 to 40 times,[16] in *P. vivax* DRAA/DGQPAG is repeated 19 times.[17,18] Among *Plasmodium* sp. there is little resemblance among the tandem repeats. In contrast, flanking the repeat region are two highly conserved regions found in CS proteins of all species called region I and region II.[16] All of the protective monoclonal antibodies (MAbs) against CS proteins recognize the central repeat regions. Furthermore, when polyclonal antibodies were produced in mice against the repeat region and the flanking conserved regions (I and II) of the *P. falciparum* CS protein, only antibodies against the repeat region blocked sporozoite invasion of hepatoma cells *in vitro*.[19] Therefore, the repeat region of the CS protein was chosen as the initial target for vaccine-induced antibodies.

B. DATA FROM ANIMAL MODEL SYSTEMS

When the gene encoding the *P. berghei* CS protein was cloned and sequenced, synthetic peptide[20-22] and *Escherichia coli*-produced recombinant protein[20] vaccines were designed to produce protective antibodies in mice against the *P. berghei* CS

protein. Protection ranging from 35[20] to 80%[22] has been achieved by immunizing with such vaccines in the *P. berghei* system, proving the principle that such vaccine-induced polyclonal antibodies can protect against sporozoite challenge. Furthermore, when the immunoglobulin from sera of mice immunized with one of these vaccines was passively transferred into naive recipient mice, three of four mice were protected, demonstrating definitively that vaccine-induced polyclonal antibodies can protect against sporozoite challenge in the absence of other parasite-specific immune responses.[20] However, in the much more infectious *P. yoelii* system, none of the vaccines designed to produce protective antibodies have ever shown any protection.[13,14] Furthermore, when *Saimiri* monkeys were immunized with vaccines designed to produce protective antibodies against the *P. vivax* CS protein repeat region, high levels of antibodies were induced, but there was no convincing evidence of protection.[23,24] Work is currently in progress in the animal model systems to more clearly define the exact B cell epitope that is the target of the protective MAbs, and to produce vaccines (containing B cell epitopes and carrier proteins for T cell help) and delivery systems (new adjuvants, liposomes, immunostimulatory complexes) that protect against malaria as consistently as does passive transfer of the MAbs. New adjuvants may serve to increase antibody responses and to maintain protective levels of antibody. Inclusion of helper T cell epitopes from malaria antigens would allow for boosting of antibody levels after natural exposure to sporozoites.[25,26] New vaccine formulations that provide prolonged release of antigen could provide their own booster doses.

C. HUMAN TRIALS

Since the gene encoding the *P. falciparum* CS protein was cloned and sequenced in 1984[16] and for the *P. vivax* CS protein in 1985,[17] there has also been considerable work done to develop and test vaccines designed to protect humans by inducing antibodies against the repeat region of the *P. falciparum* and *P. vivax* CS proteins. There has been some work in the *P. vivax* system,[27] but the vast majority of work has been in the *P. falciparum* system. In the first studies an *E. coli*-produced recombinant vaccine called $R32tet_{32}$ or FSV-1,[28] and a synthetic peptide vaccine were tested.[29] $R32tet_{32}$ included 32 tetrapeptide repeats from the repeat region of the *P. falciparum* CS protein, $(NANP)_{15}NVDP(NANP)_{15}NVDP$, (R32) (B cell epitopes, the target or business end of the vaccine), fused to 32 amino acids from the tetracycline resistance region of a plasmid (T helper cell epitopes, the carrier protein); and was delivered with aluminum hydroxide as adjuvant (the delivery system). The second vaccine included three copies of NANP (B cell epitopes) conjugated to tetanus toxoid (T helper cell epitopes) and delivered with aluminum hydroxide as adjuvant. In both cases only a few of the individuals immunized had expected levels of antibodies; and one of six individuals challenged in one trial,[28] and one of three individuals challenged in the other trial[29] were protected. In both studies other individuals with high levels of antibodies had delays in the onset of parasitemia indicating that at least 95% of sporozoites had been rendered ineffective by the vaccine-induced antibodies, and it appeared that individuals with the highest levels of antibodies were the ones protected. Since both R32 and $(NANP)_3$ seemed capable

of inducing protective antibodies, subsequent work in humans has focused primarily on varying the carrier protein for providing T cell help and the delivery system so as to provide better interaction among the B lymphocytes, T helper lymphocytes, and antigen-presenting cells required to consistently produce high levels of protective antibodies. These antibodies must be superior to those induced by natural infection, since naturally acquired antibodies to the repeat region of the CS protein were not associated with resistance to reinfection in a study in Kenya.[30] *Pseudomonas aeruginosa* toxin A,[31] the nonstructural protein of influenza A,[27,32,33] tetanus toxoid,[29] meningococcal outer membrane protein,[34] choleragenoid,[34] hepatitis B virus surface antigen,[35] flanking regions of the CS protein,[36] and carboxy terminus[36a] of the CS protein have all been tested as carrier proteins. A combination of monophosphoryl lipid A and cell wall skeleton of mycobacteria (Detox™),[32] liposomes with aluminum hydroxide and monophosphoryl lipid A,[33] hepatitis B surface antigen particles with aluminum hydroxide and monophosphoryl lipid A,[36a] and interferon-α[37] have all been tested as adjuvants. A number of these have proved promising, with 18[37a] to 25%[36a] of volunteers completely protected against malaria, and equivalent percentages showing a significant delay in the onset of parasitemia indicating that greater than 95% of sporozoites have been inactivated.

Thus, in 1992 15 to 25% of volunteers can consistently be completely protected against malaria by vaccines designed to produce protective antibodies against the repeat region of the *P. falciparum* CS protein. Work is now in progress to improve this level of protection by varying B and T cell epitopes and delivery systems. One of the most promising approaches is the use of multiple antigen peptides (MAP);[38] and in the near future such vaccines, perhaps delivered in liposomes with aluminum hydroxide and monophosphoryl lipid A, will undergo clinical trials. Another approach is to induce antibodies against the flanking regions of the CS protein in addition to antibodies against the repeat region. Regions flanking the repeats may play a role in the binding of sporozoites to hepatocytes (see below), and antibodies against region II inhibit *P. berghei* invasion of hepatocytes *in vitro*.[39] Sera from some individuals living in malaria endemic areas have antibodies that recognize epitopes in the nonrepeat region.[40,41] Ultimately vaccines will require extensive field testing to determine whether they are effective in the field and to determine whether natural infection will boost the antibody responses to the vaccines. Preliminary studies from Thailand suggest that antibodies induced by immunization with a vaccine that only includes *P. falciparum* sequences from the repeat region (R32) can be boosted by natural exposure,[41a] just as they were in mice.[25] Regardless of whether there will be improvement on the 15 to 25% complete protection now achieved by these vaccines, it is certain that the repeat region of the CS protein will be one component of a multicomponent vaccine designed to produce humoral and cellular immune responses against a number of targets (see below).

D. IDENTIFICATION OF OTHER TARGETS FOR PREVENTING EFFECTIVE SPOROZOITE INVASION OF HEPATOCYTES

The mechanism whereby sporozoites attach to and invade hepatocytes is unknown. In 1986, Aley and colleagues[42] reported that a region 5′ of the repeat of

the *P. falciparum* CS protein was involved in binding of sporozoites to hepatocytes. More recently Pancake et al.[43] and Cerami et al.[39,44] have suggested that sporozoites bind to hepatocytes via adhesion motifs in the region II area of the CS protein that recognize sulfated glycoconjugates on hepatocyte membranes. Interestingly, a non-CS sporozoite surface protein, the sporozoite surface protein 2 (SSP2), contains a sequence homology with region II.[45-47] Others have been working on the identification of ligands and receptors on sporozoites and hepatocytes.[48] This is an area of enormous potential importance, and it is hoped that someday vaccines will induce antibodies that recognize functional regions on sporozoites and prevent infection by interfering with the interaction of these regions with hepatocytes.

III. ATTACKING THE INFECTED HEPATOCYTE

A. IRRADIATED SPOROZOITE VACCINE-INDUCED PROTECTIVE IMMUNITY DEPENDENT ON CD8$^+$ T CELLS

Monoclonal antibodies against the CS protein repeat region protect mice[12,14,20] and monkeys[15] against sporozoite-induced malaria, yet there is now a strong body of evidence indicating that the immunity induced by the irradiated sporozoite vaccine is mediated by T cells that recognize malaria peptides presented in the context of class I MHC molecules on infected hepatocytes. The potential role of T cells in this immunity was first recognized by Chen and colleagues[49] who showed that 64% of μ-suppressed mice who were immunized with irradiated sporozoites were protected, indicating that antibodies were not required for this protection.[49] In 1987 it was shown that adoptive transfer of immune T cells into naive mice protected against malaria in the absence of antibodies,[20] and then Schofield and colleagues[50] and Weiss and colleagues[51] working in the A/J-*P. berghei* and Balb/c-*P. yoelii* system showed that protective immunity induced by immunization with irradiated sporozoites was abrogated by *in vivo* depletion of CD8$^+$ T cells; the antibodies induced by immunization with irradiated sporozoites were not adequate to protect against sporozoite-induced malaria, and the CD8$^+$ T cells were required. Depletion of CD4$^+$ T cells had no effect on protection. These data strongly suggested that this immunity was dependent on CD8 + cytotoxic T lymphocytes (CTL) recognizing malaria antigens presented on infected hepatocytes. However, the majority of liver stage schizonts in naive animals developed normally with no evidence of inflammatory cells.[52] Furthermore, there were major questions regarding whether hepatocytes expressed class I MHC molecules; and whether T cells could pass from the sinusoids through Kupffer cells, through the space of Disse, and attack infected hepatocytes. Hoffman and colleagues[52] reported that when mice immunized with irradiated *P. berghei* sporozoites were challenged with large numbers of live sporozoites, they developed parasite-specific CD8$^+$ T cell-dependent inflammatory infiltrates in the livers. They also showed that spleen cells from mice immunized with irradiated sporozoites eliminated infected hepatocytes from *in vitro* culture in an MHC-restricted and species-specific manner,[52] indicating that immune T cells were recognizing *Plasmodium* sp. antigens presented on infected hepatocytes and eliminating these cells. Since this activity was not reversed by anti-IFN-γ and not

duplicated by culture supernatants, it was thought to be mediated through direct T cell-hepatocyte interaction by CTL. Although it appeared that an important mechanism of irradiated sporozoite protective immunity had been established, the targets still remained undefined.

B. CD8+ CYTOTOXIC T LYMPHOCYTES AGAINST THE CS PROTEIN ARE PROTECTIVE IN ADOPTIVE TRANSFER

In the late 1980s the only target available for study was the CS protein. Romero and colleagues[53] working in the *P. berghei* system and Weiss and colleagues[54] working with *P. yoelii*[54] reported that there was only a single region of these rodent malaria CS proteins that included a CTL epitope. CTL against the *P. yoelii* epitope eliminated infected hepatocytes from culture in an antigen-specific, MHC-restricted manner;[54] and a CTL clone against the analogous region of the *P. berghei* CS protein adoptively transferred complete protection against challenge with *P. berghei* sporozoites.[53] It was subsequently shown that transfer of a similar CD8+ CTL clone against the *P. yoelii* CS protein transferred protection[55,56] and that if this CD8+ CTL clone was transferred 3 h after sporozoite inoculation, it still provided protection. Since inoculated sporozoites are not accessible to antibodies within 5 min of inoculation and are thought to enter hepatocytes within an hour of inoculation, this experiment indicated that the CTL clones were recognizing CS protein expressed in infected hepatocytes and either destroying the infected hepatocyte or rendering the parasite nonfunctional. This concept was further supported by data demonstrating that radiolabeled protective, but not nonprotective CTL clones could be found in apposition to infected hepatocytes after adoptive transfer *in vivo*.[57] The mechanism whereby these CD8+ T cells prevent further development of the parasites is unknown. They may act through the release of pore-forming proteins or cytokines. There is also evidence that they may require specific adhesion molecules such as CD44 on their surfaces to optimally interact with infected hepatocytes.[57]

C. IMMUNIZATION WITH CS PROTEIN VACCINES INDUCES CD8+ T CELL-DEPENDENT PARTIAL PROTECTION IN RODENT MALARIA MODEL SYSTEMS

There have been efforts to produce vaccines that actively induce CTL against the CS protein. In the *P. berghei* system it was shown that oral immunization of mice with a recombinant *Salmonella typhimurium* expressing the *P. berghei* CS protein induced CTL against the *P. berghei* CS protein,[58] and protected 50 to 75% of mice against challenge with *P. berghei* sporozoites.[58,59] As with the irradiated sporozoite vaccine, this immunity was abrogated by *in vivo* depletion of CD8+ T cells.[58] When mice were immunized with a recombinant vaccinia virus expressing the *P. berghei* CS protein, they produced CTL against the *P. berghei* CS protein and were not protected.[60] Likewise, mice immunized with recombinant vaccinia, *S. typhimurium*, or pseudorabies virus expressing the *P. yoelii* CS protein produced excellent cellular immune responses, but were not protected against the highly infectious *P. yoelii* sporozoites.[61-63] However, when Balb/c mice were immunized with irradiated P815 mastocytoma cells transfected with the gene encoding the *P.*

yoelii CS protein, the mice produced CTL against the CS protein; and 50 to 85% was protected against challenge.[64] Like the immunity found after immunization with irradiated sporozoites, this protective immunity was eliminated by *in vivo* depletion of $CD8^+$ T cells.[64]

D. DEVELOPMENT OF VACCINES TO INDUCE CTL AGAINST THE *P. FALCIPARUM* CS PROTEIN IN HUMANS

This strong body of data from the rodent malaria system has turned the attention of vaccine developers toward producing human vaccines that induce CTL against the *P. falciparum* CS protein. The first step in this process was the identification of CTL epitopes on the *P. falciparum* CS protein. This was first accomplished in 1988 by Kumar and colleagues[65] who demonstrated $CD8^+$ T cell-dependent cytolytic activity against a 23-amino acid region on the *P. falciparum* CS protein, Pf 7G8 CS 368–390, in B10.BR mice. Malik and colleagues,[66] using peripheral blood mononuclear cells (PBMC) from volunteers immunized with irradiated *P. falciparum* sporozoites, were able to demonstrate that these volunteers had $CD8^+$ T cell-dependent cytolytic activity against the same region, Pf 7G8 CS 368–390.[65] Subsequently Sedegah and colleagues[67] showed that Kenyans with life-long natural exposure to malaria also had circulating $CD8^+$ CTL against the same region. Doolan and colleagues[68] have demonstrated that PBMC from Australians who had lived in malarious areas also had cytolytic activity against a similar region of the CS protein, but did not demonstrate genetic restriction of the response or T cell subset dependence of the activity. With the establishment of an assay for identifying such CTL, a number of groups are studying the capacity of soluble recombinant proteins and recombinant live vectors such as *Salmonella typhi*, vaccinia, and bacille Calmette-Guérin (BCG) expressing the CS protein to induce CTL against the CS protein. In the next few years there should be abundant information regarding induction of $CD8^+$ CTL against the CS protein in humans. In parallel there will be considerable work required to consistently protect mice using vaccine constructs and delivery systems that can be applied in humans. Several modifications of vaccine construction and delivery systems are currently being evaluated. For example, MAPs constructed of peptides representing T helper and CTL epitopes, and MAPs or peptides mixed with novel adjuvants or incorporated in liposomes and immunostimulatory complexes are being explored as possibilities. Immunization with antigens in liposomes or immunostimulatory complexes could induce CTL, thereby eliminating the need for using live vaccine vectors in human subjects.

E. IDENTIFICATION AND DEVELOPMENT OF SPOROZOITE SURFACE PROTEIN 2 AS A TARGET OF VACCINE-INDUCED $CD8^+$ PROTECTIVE CTL

Immunization with irradiated sporozoites completely protects against malaria, but none of the subunit *P. berghei* or *P. voelii* CS protein vaccines have given protection comparable to the irradiated sporozoite vaccine. Furthermore, in the human studies the presence of CTL against the *P. falciparum* CS protein did not guarantee that the individual would be protected; and likewise, one individual who

was not shown to have CTL was protected against challenge.[9,66] Considering the complexity of sporozoites it was not logical to assume that all protection induced by the whole organism vaccine was mediated by CTL against a single short stretch of amino acids on a single protein. Thus, there has been considerable effort to identify additional targets of irradiated sporozoite-induced protection immunity. Charoenvit and colleagues[69] immunized mice with irradiated *P. yoelii* sporozoites and produced a monoclonal antibody directed against a 140-kDa sporozoite protein.[69] The gene encoding this protein was cloned and sequenced,[46,47] and the protein was named sporozoite surface protein 2. To determine whether immunization with irradiated sporozoites not only produced antibodies, but also CTL against SSP2, Khusmith and colleagues[64] transfected a 1.5-kb fragment of the gene encoding SSP2 into P815 mouse mastocytoma cells that could be used as targets in CTL assays and showed that mice immunized with irradiated sporozoites produced CTL against SSP2.[64] Khusmith subsequently produced $CD8^+$ CTL clones against SSP2 and showed that adoptive transfer of one of these clones completely protected against challenge, establishing that CTL against SSP2 could completely protect against this highly virulent parasite in the absence of any other parasite-specific immune responses.[69a] Mice were then immunized with the P815 cells expressing *P. yoelii* SSP2 (PySSP2); approximately 50% was protected against challenge, and the immunity was dependent on $CD8^+$ T cells.[64] The gene encoding the *P. falciparum* SSP2 (PfSSP2) has now been identified and characterized,[70] and shown to be the previously described thrombospondin-related anonymous protein (TRAP).[45] Work is now in progress to produce human vaccines that will induce protective CTL and perhaps antibodies against SSP2.

F. IMMUNIZATION WITH CS PLUS SSP2 GIVES ADDITIVE PROTECTION

At this point it had been shown that immunization with the PyCS protein or PySSP2 vaccines gave only partial protection against malaria (50 to 75%), immunity that was in no way comparable to the complete protective immunity found after immunization with irradiated sporozoites. Khusmith and colleagues[64] then immunized with transfected P815 cells expressing PyCSP and PySSP2 and achieved 100% protection. Furthermore, as is observed following immunization with irradiated sporozoites this immunity was completely reversed by *in vivo* depletion of $CD8^+$ T cells. Humans cannot be immunized with tumor cells expressing malaria antigens, and thus there is a major effort to develop antigen delivery systems that can be used in humans that provide comparable protection.

G. $CD4^+$ CTL AGAINST THE CS PROTEIN MEDIATE PROTECTIVE IMMUNITY

Since the seminal observations by Schofield et al. and Weiss et al. demonstrating the $CD8^+$ T cell dependence of irradiated sporozoite-induced protective immunity, there has been a major emphasis on $CD8^+$ CTL. Recently, however, Renia and colleagues[71] have demonstrated that $CD4^+$ T cells directed against amino acids 59–79 from the amino terminus of the *P. yoelii* CS protein can recognize CS protein peptides presented on infected hepatocytes, eliminate infected hepatocytes from culture, and adoptively transfer protection against malaria. Accordingly work is in

progress to actively induce such protective immunity in the rodent model systems, and to identify analogous regions of the *P. falciparum* and *P. vivax* CS proteins so as to construct vaccines for humans. Recently, Moreno and colleagues[72] reported that immunization of a human with *P. falciparum* sporozoites induced cytotoxic $CD4^+$ T cells that recognized an epitope in the C-terminal region of the CS protein.

H. IDENTIFICATION OF ADDITIONAL TARGETS OF PROTECTIVE IMMUNE RESPONSES IN INFECTED HEPATOCYTES

There are several perspectives among investigators working to discover important liver stage antigens. Since immunization with irradiated sporozoites provides such potent protective immunity, one approach is to limit the investigation to antigens that are present at the latest stage of development of irradiated sporozoites in hepatocytes, and against which antibody or T cell responses are induced by immunization with irradiated sporozoites. Both the CS protein and PySSP2, which are present in sporozoites and infected hepatocytes, were discovered using this approach. The second approach is to look for any antigen expressed in infected hepatocytes, regardless of its role in irradiated sporozoite-induced protective immunity, and assess its capacity to induce protective immune responses. A *P. falciparum* liver stage-specific protein, liver stage antigen-1 (LSA-1);[73,74] a *P. berghei* liver stage-specific protein, *P. berghei* liver 1 (PbL1);[75] a 230-kDa *P. berghei* liver stage antigen, LSA-2;[76] and a 17-kDa *P. yoelii* liver and blood stage protein, LISA-3[75a] have all been discovered using the second approach. Thus far there have been no published reports indicating that any of these proteins contribute to the protective immunity induced by immunization with irradiated sporozoites.

Using sera from individuals who had spent long periods of time in Africa taking chemoprophylaxis against malaria, Guerin-Marchand and colleagues[73] identified and sequenced a DNA fragment of 196 base pairs (bp) composed entirely of 51-bp repeat sequences that they called LSA-1. This 51-bp repeat encodes a highly conserved 17-amino acid repeat polypeptide.[73] The complete gene structure and the LSA-1 protein sequence (230 kDa) were published subsequently by Zhu and Hollingdale.[74] The biological activity of antibodies or T cells directed against this protein has not been reported.

PbL1 was identified using a MAb generated by immunizing mice with *P. berghei*-infected hepatoma cells I.[75] The MAb, anti-PbL1, is specific to the exoerythrocytic stage and does not react with the sporozoite or the blood stage. Passive transfer *in vivo* of the MAb did not protect against a challenge of sporozoites; however, a reduction of the parasitemia was observed.[77] The MAb did not affect the growth of liver stage cultures *in vitro*.[77]

Hollingdale and colleagues[76] reported that antiserum to the *P. falciparum* LSA-1 peptide recognized a novel 230-kDa antigen of *P. berghei* liver exoerythrocytic schizonts, namely, LSA-2. Fluorescence labeling was not detected before 24 h after sporozoite invasion. Immunoelectron microscopy has localized this antigen on the parasitophorous vacuole membrane of 50-h-old parasites.[78] By transmission electron microscopy they described isolated labeled vesicles located at the periphery of the infected hepatoma cell. Because of the latter observation and since the molecular

weight of PbL1 is not reported, the authors suggested a possible relationship between PbL1 and the 230-kDa LSA-2,[78] and reported preliminary work indicating that immunization with an LSA-2 peptide protected mice against *P. berghei* infection.[76]

Charoenvit and colleagues[78a] immunized mice with infected hepatocytes from mice challenged with large numbers of *P. yoelii* sporozoites 43 h previously and produced a monoclonal antibody, Navy yoelii liver stage 3 (NYLS3) that does not recognize sporozoites, but recognizes infected hepatocytes and erythrocytic stage parasites. This antibody has direct activity against infected hepatocytes, and work is in progress to clone the gene encoding this protein (called LISA-3) and to identify the *P. falciparum* homologue of LISA-3.

Furthermore, the *P. falciparum* major merozoite surface protein-1 (MSP-1) was shown a number of years ago to be expressed in late liver stage schizonts,[78b] and recently the 175-kDa *P. falciparum* erythrocyte-binding antigen (EBA-175) has been shown to be present in liver stage schizonts.[78c] It may be that these or other "blood" stage antigens that are also "liver" stage antigens are the targets of protective antibody and T cell responses when expressed in infected hepatocytes.

Despite considerable effort and identification of multiple liver stage proteins, none of these proteins have been consistently shown to be involved in protective immune responses *in vivo*. Further work will clarify the potential importance of LSA-1, PbL1, LSA-2, and LISA-3; and perhaps these proteins and yet undiscovered liver stage proteins will be included in multivalent preerythrocytic stage vaccines.

I. INTERFERON-γ AND OTHER CYTOKINES

The mechanisms by which $CD4^+$ and $CD8^+$ T lymphocytes actually eliminate infected hepatocytes from culture and protect *in vivo* are not well defined; however, cytokines may play a role. Systemic administration of interferon-γ partially protects mice and monkeys against *P. berghei*[79] and *P. cynomolgi*,[80] respectively; and *in vitro* treatment of infected hepatocytes with interferon-γ eliminates *P. falciparum*[81] from culture. The protective immunity induced by irradiated *P. berghei* sporozoites in A/J mice was abrogated by *in vivo* treatment of the mice with anti-interferon-γ.[50] This was not found in *P. berghei*-[52] or *P. yoelii*-[55]immunized Balb/c mice. However, recently it has been shown that adoptive transfer of a $CD8^+$ T cell clone against the *P. yoelii* CS protein that endogenously produces large quantities of interferon-γ protects against *P. yoelii*, and this protective immunity is eliminated by *in vivo* treatment of the mice with anti-interferon-γ.[56] It is still not clear how vaccines will be designed so as to produce protective interferon-γ responses, and it may be that the protective *P. berghei*[59] and *P. yoelii*[74] vaccines already tested lead to the local release of protective levels of interferon-γ

Analysis of the pattern of secretion of certain CD4 T cell clones suggests that other cytokines could be involved.[82] The inhibitory effect of IL-1 and IL-6 on intrahepatic development of human and murine parasites has been reported.[81,83] Tumor necrosis factor (TNF) inhibited development of *P. berghei in vitro* in a hepatoma cell line,[84] but TNF was not effective alone in primary cultures of *P. yoelii*-infected hepatocytes.[85,86] However, in cocultures of hepatocytes and non-parenchymal cells, TNF induced parasite inhibition by IL-6 release.[86,87] The mech-

anism by which cytokines kill infected hepatocytes is not well established; however, recent reports indicate that interferon-γ and perhaps other cytokines induce infected hepatocytes to produce L-arginine-derived nitrogen oxides that are toxic to the intracellular parasite.[85,87]

IV. THE FUTURE: INDUCING MULTIPLE IMMUNE RESPONSES AGAINST MULTIPLE TARGETS

Vaccines that are intended to induce protective immune responses against the preerythrocytic stages of the parasite are designed to completely protect against malaria; or if used in combination with an erythrocytic stage vaccine, to substantially reduce the number of inoculated sporozoites that develop to mature liver stage schizonts and release infective merozoites. We know that this type of immunity can be achieved because immunization of humans with radiation-attenuated *P. falciparum* sporozoites consistently protects against challenge. It seems logical that the protective immunity induced by the attenuated "whole organism" vaccine must be directed against multiple targets and mediated by multiple immune mechanisms. We know that monoclonal antibodies against the repeat region of the CS protein expressed on the surface of circulating sporozoites; and $CD8^+$ CTL against a single epitope in the carboxy terminus of the CS protein, $CD4^+$ CTL against a single epitope in the amino terminus of the CS protein, and $CD8^+$ CTL against a single epitope on the PySSP2, all presumably expressed in infected hepatocytes, can all completely protect against sporozoite-induced malaria in the absence of other parasite-specific immune responses. Furthermore, antibodies against an antigen first expressed in infected hepatocytes (LISA-3 in the *P. yoelii* system) eliminate infected hepatocytes from culture, presumably by recognizing this protein expressed in infected hepatocytes.[87a] Thus, five discrete targets on the sporozoite and infected hepatocytes, and at least three different types of immune responses have been shown to be associated with protective immunity. It is likely that the irradiated sporozoite vaccine actually induces additional protective immune responses against additional targets, and work is in progress to identify these targets and mechanisms. Nonetheless, it seems immediately apparent that a coherent strategy would be to try to produce vaccines for humans that induce these varied responses, and such work is in progress.[88]

There are several ways of conceptualizing how such vaccines might work. In one scenario, if 100 sporozoites were inoculated by a single mosquito bite, it is possible that 80% of those sporozoites would be eliminated or rendered noninfectious by antibodies to the repeat region of the CS protein. There would then be 20 sporozoites developing within hepatocytes. If 75% of these developing liver stage parasites was eliminated by CTL against CS protein, there would still be five parasites developing within hepatocytes. If 80% of the five was eliminated by CTL against SSP2, there would still be one parasite developing to a mature liver stage schizont, and the person would become infected just as though none of the other immune responses had been invoked. If, however, the last parasite is eliminated by antibodies against the human malaria analog of LISA-3, then the individual will

be protected. Thus, a multivalent vaccine could be effective by erecting a series of incomplete barriers to the parasite and by leading to the last barrier (immune response) being challenged with a markedly reduced parasite load, resulting in complete protection.

Another way to look at a multivalent vaccine is to consider that among 100 individuals, perhaps 20% will be "completely" protected by antibodies against the repeat region of the CS protein, something that we are already able to do. Another 30% may be completely protected by CTL against CSP, another 30% by CTL against SSP2, and 20% by antibodies against the human malaria analog of LISA-3. Thus, by delivering an effective multivalent vaccine everyone would be protected. In fact, it is more likely that a matrix of these two conceptualizations will more closely approximate reality; and that is what we are eventually expecting from a multivalent vaccine.

During the past decade there have been enormous advances in our understanding of the mechanisms and targets of irradiated sporozoite-induced protective immunity.[88] There will undoubtedly be numerous problems in the future constructing human vaccines that induce the required immune responses against the targets that have been identified. They include development of methods for optimal vaccine construction and delivery so as to maximize required immune responses against multiple targets. Once effective vaccines are developed, the question of expense of production and delivery will also have to be addressed if such vaccines are ever to be available to the people who need them most. Nonetheless, there is now great hope that we will one day have vaccines to protect against malaria by attacking the parasite at multiple stages in its preerythrocytic cycle, and that such vaccines will be combined with vaccines to attack the asexual and sexual erythrocytic stages of the parasite.

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